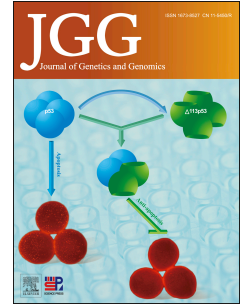


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Potential and progress of studying mountain biodiversity by means of butterfly genetics and genomics

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1 **Potential and progress of studying mountain biodiversity by means of butterfly genetics**

2 **and genomics**

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13 **ABSTRACT**

14 Mountains are rich in biodiversity, and butterflies are species-rich and have a good ecological
15 and evolutionary research foundation. This review addresses the potential and progress of
16 studying mountain biodiversity using butterflies as a model. We discuss the uniqueness of
17 mountain ecosystems, factors influencing the distribution of mountain butterflies,
18 representative genetic and evolutionary models in butterfly research, and evolutionary studies
19 of mountain biodiversity involving butterfly genetics and genomics. Finally, we demonstrate
20 the necessity of studying mountain butterflies and propose future perspectives. This review
21 provides insights for studying the biodiversity of mountain butterflies as well as a summary of
22 research methods for reference.

23

24 **Keywords:** Mountain ecosystem, Butterfly, Biodiversity, Adaptation, Genomics, Genetics

25 **Introduction**

26 Biodiversity is an ecological concept used to describe the extent of diversity in nature and can
27 be characterized at multiple levels, such as genetic diversity, species diversity, and ecosystem
28 diversity. Studies of biodiversity provide insights into the relationships between different
29 taxonomic groups, such as terrestrial vertebrates and vascular plants (Kier et al., 2009), as well
30 as between anthropogenic activities and ecosystems (Hautier et al., 2015). Notably, in terrestrial
31 biogeographic regions, many vascular plant and vertebrate species are distributed in areas that
32 occupy very little of the Earth's land surface, i.e., biodiversity hotspots (Myers, et al., 2000;
33 Mittermeier et al., 2004; Mittermeier et al., 2011; Noss et al., 2015). Among these biodiversity
34 hotspots, mountain ecosystems harbor rich and diverse terrestrial biodiversity. While mountains
35 cover only 25% of the world's land area (excluding Antarctica), mountains and adjacent
36 lowlands harbor approximately 87% of amphibian, bird, and mammal species, a large number
37 of which are endemic to mountains (Rahbek et al., 2019a). Assessments of biodiversity hotspots
38 based on plant diversity have also shown that mountains possess the highest diversity (Myers
39 et al., 2000), with most biodiversity hotspots containing mountainous regions (Fig. 1A). All
40 these findings indicate the importance of mountain ecosystems. However, the biodiversity
41 research has focused on vertebrates or vascular plants, resulting in a "megafauna/flora bias".
42 Although insects are the most species-rich group in the animal kingdom and play an important
43 role in the whole ecosystem and in human life, there is a lack of understanding of general
44 patterns of insect diversity (Basset et al., 2012; Ashton et al., 2016; ; Szewczyk and McCain,
45 2016; Beck et al., 2017).

46 As a representative group of insects, butterflies, with over 18,700 described species, are
47 probably the most well-known taxonomic group (Thomas, 2005; van Nieukerken E, et al., 2011)
48 and are widely distributed. They have received considerable attention because of their high
49 phenotypic diversity and richness of species. In addition, butterflies depend exclusively on host
50 plants and niches, so they are sensitive to climatic and environmental changes (Oliver et al.,
51 2015). Additionally, butterflies are important pollinating insects for plants. For these reasons,
52 butterfly monitoring schemes have been established in several countries and regions, such as
53 the European Butterfly Monitoring Scheme (eBMS, <https://butterflymonitoring.net/>) and China
54 Biodiversity Observation Network (China BON, [https://geobon.org/bons/national-regional-
55 bon/national-bon/China-bon/](https://geobon.org/bons/national-regional-bon/national-bon/China-bon/)). Additionally, when we conducted a rough query of publications
56 by searching “mountain butterfly” or “montane butterfly” on Web of Science
57 (<https://www.webofscience.com>) and complemented these results by searching for some
58 specifically studied genera including mountain butterflies, such as *Heliconius*, *Danaus*,
59 *Melitaea*, *Erebia* and *Kallima*, we noticed an increasing tendency—yet still with potential for
60 development—of publication records related to mountain butterflies published during the past
61 two decades (publication records/year: 58/2000, 102/2005, 148/2010, 188/2015, 244/2020). In
62 addition to the growing attention to butterfly diversity, rapid advances in multi-omics and
63 genetics in recent years have provided new methods for studying mountain butterflies, such as
64 phylogenomics (Van Belleghem et al., 2017; Edelman, N.B. et al., 2021; Wang et al, 2022),
65 comparative genomics (Zhan et al., 2011; The *Heliconius* Genome Consortium, 2012),
66 population genomics (Reed et al., 2011; Kunte et al., 2014; Nadeau et al., 2014, 2016; Zhan et
67 al., 2014; Van Belleghem et al., 2017; Wang et al., 2022) and genome editing (Mazo-Vargas et

68 al., 2017, 2022; Zhang et al., 2017), which help us to delve into and understand the relationships
69 between environmental factors and biological processes from a wider range of perspectives.
70 These features position butterflies not only as important indicator species but also good models
71 for addressing evolutionary and developmental questions.

72 In this review, we summarize the progress and explore the potential of studying mountain
73 biodiversity in multiple dimensions using butterflies as a model. At the species diversity level,
74 we explore the overall causes of high diversity in mountain ecosystems from a macro
75 perspective to provide the basis for related butterfly studies. Then, we discuss the factors that
76 influence the richness of mountain butterfly distributions with examples from three types of
77 diversity parameters and reveal the gaps in butterfly inventories across the world. We also
78 provide some cases of studying genetic diversity based on genetic loci to lead the review to a
79 micro perspective. Third, we present butterfly models that have been systematically
80 characterized to provide ideas for the in-depth study of butterfly characteristics with advanced
81 analysis and experimental tools in the genomic era. Fourth, we summarize recent studies that
82 elucidate the evolutionary mechanisms of mountain butterfly biodiversity by means of genetics
83 and genomics. Finally, we offer some thoughts about future research directions including
84 enhancing surveys on biodiversity, conducting multi-omics studies to address evolutionary or
85 adaptive mechanisms of butterflies in montane biotas.

86

87 **The uniqueness of mountain ecosystems and the need for related research**

88 A straightforward way to understand the causes of the high biodiversity of mountains is to
89 investigate correlations between species richness and other factors (both biotic and abiotic).

90 Throughout the history of ecology, researchers have been attempting to address this issue using
91 various species at different spatial scales. For example, at the 1° resolution, topography and
92 temperature are the most important global predictors of avian richness in multi-predictor
93 models, while from a global perspective, mountain ranges in high-energy areas become
94 primarily important (Davies et al., 2007). Data on vascular plants show that potential
95 evapotranspiration, the number of wet days per year, and measurements of topographical and
96 habitat heterogeneity are core predictors of species richness (Kreft and Jetz, 2007). However,
97 it is difficult for survey-based analyses to explain the extraordinary species richness in tropical
98 mountain regions (Rahbek and Graves, 2001; Kreft and Jetz, 2007; Rahbek et al., 2007).
99 Therefore, theoretical models have also been developed to answer this question (Doebeli and
100 Dieckmann, 2003; Graham et al., 2004; Nicholas et al., 2009; Connolly et al., 2017). For
101 example, Rangel et al. (2018) simulated the evolution of South America over the past 800,000
102 years by incorporating spatial, temporal, physical and biological factors. Although the
103 simulations had no target patterns and no empirical data, the results showed striking similarities
104 to contemporary distribution patterns of birds, mammals, and plants, confirming the role of
105 topography and climate in driving evolution and diversification (Rangel et al., 2018). This study
106 emphasized factor evaluation from an evolutionary perspective, which implies that current
107 biodiversity is the result of historical ecological events. In recent years, a growing number of
108 studies have demonstrated and summarized the significance of geological and climatic
109 influences in shaping mountain biodiversity (Antonelli et al., 2018; Rahbek et al., 2019a,
110 2019b).

111 As a fundamental driver of biodiversity patterns, climate can influence biodiversity from

112 various aspects. First, the great variations in altitude help to establish a collection of diverse
113 climates, which facilitates the diversification of animal habitats (Körner, 2004). Second, both
114 short-term and long-term climatic oscillations can act differently in mountainous areas than in
115 plains (Rahbek et al., 2019a). Third, some small valleys may hold climatic pockets, which may
116 serve as refugia for organisms (Bennett et al., 1991; Shafer et al., 2011; Valencia et al., 2016).
117 The diversity and uniqueness of mountain climates provide favorable conditions for speciation
118 and species coexistence, thus increasing biodiversity.

119 In terms of geological history and evolution, the uniqueness of mountain areas also has an
120 impact on biodiversity. Orogenic movements have led to the accumulation of strata of different
121 ages, origins, and compositions, resulting in a high degree of heterogeneity in mountain rocks
122 and topography, which influence species diversification through periodic "build, join, and
123 disappear" processes (Craw et al., 2016). Echoing this, evolutionary radiations are often
124 associated with mountain uplift (Favre et al., 2015). In addition, the process of mountain
125 building may screen specific communities by altering the local climate (Antonelli et al., 2018).

126 In practical studies, researchers need to combine the dynamic processes of both climate change
127 and geological factors to contrast the processes of diversification and to distinguish whether
128 diversity arises due to speciation in mountains or whether mountains are a refuge for species.

129 This direction has also become a multidisciplinary subject in biology, geology, and climatology.
130 For example, the most abundant temperate alpine flora is distributed on the Qinghai-Tibet
131 Plateau, the Himalayas, and the Hengduan Mountains (Li et al., 2014), also known as the Tibet-
132 Himalaya-Hengduan region (THH region). Rates of biotic assembly revealed that extant
133 lineages first diversified in the Hengduan Mountains during the early Oligocene, and then their

134 accelerated diversification and colonization in THH regions were likely driven by orogeny and
135 the intensification of the Asian monsoon (Ding et al., 2020). The uniquely rich flora constructed
136 diverse habitats for fauna such as vertebrates and invertebrates. In conclusion, the above
137 macroscopic research advances provide the basis for studies using butterflies as models and
138 help to further elucidate the mystery of mountain biodiversity at the genetic and genomic levels.

139

140 **Butterfly diversity and factors affecting butterfly distribution in mountainous areas**

141 As mentioned above, an understanding of the global patterns of butterfly diversity that may
142 serve as an important reference framework for butterfly research is still lacking. Community
143 science observation activities and records could help aid in this process. For example, the
144 Global Biodiversity Information Facility (GBIF), Integrated Digitized Biocollections (iDigBio),
145 eButterfly, and iNaturalist are well-developed platforms. The data provided by naturalists on
146 these platforms are important resources for tracking temporal and spatial variations in
147 butterflies (Kral-O'Brien et al., 2021; Davis et al., 2022). Here, we filled in gaps in the
148 assessment of butterfly inventories at a global scale based on the latest GBIF records via the
149 same method applied by Girardello et al. (2019) (Fig. 1B). Compared with previous findings,
150 our updated results show that in Europe, North America, the coastal regions of Australia and
151 Southern Africa, inventory completeness remains high, while most inventory gaps can be
152 observed in South America, central Africa, Asia, and Oceania (excluding Australia and New
153 Zealand), the inventories of which have barely changed. Moreover, there is a paucity of
154 butterfly inventories in some biodiversity hotspot regions, such as the Indo-Burma, Himalaya,
155 and Tropical Andes (Fig. 1B). Despite this, the accumulation in the number of observations in

156 these regions has accelerated in recent years, which mirrors the increasing interest in butterfly
157 diversity, especially in mountainous regions or reserves (Girardello et al., 2019). The species
158 distribution data help assess the conservation status of butterflies and reveal spatial biases at
159 the global scale.

160 In the context of butterfly diversity, studies can be generally divided by their focus. Some
161 studies have aimed to reveal the determinants of species richness at local or landscape scales
162 (α or γ diversity), while others have sought to examine variations in β diversity to discover
163 community structure (**Box 1**). For species richness, a hump-shaped pattern along altitudinal
164 gradients has been observed (Pyrzcz and Wojtusiak 2002; Wilson et al., 2007; Kumar et al.,
165 2009), implying that butterfly species richness peaks at intermediate elevations. This
166 phenomenon has also been found in other invertebrates and mammals (McCoy, 1990; McCain,
167 2005), which can be explained by the overlap of species from low and high altitudes and the
168 increase in niche types. However, the results of studies in some areas showed a monotonic
169 decrease or increase in species richness with elevation (Wettstein and Schmid 1999; Pellissier
170 et al., 2012; Leingärtner 2014), which can be attributed to the biology of the studied insects or
171 to the disturbance of human activities at the foothills (Nogués-Bravo et al., 2008). In addition,
172 the topographic features of mountainous areas may also influence distribution patterns. For
173 example, the variance in the butterfly richness pattern between Olympus and Rhodopes was
174 ascribed to the differences in topography, with Olympus being higher and steeper (Kaltsas et
175 al., 2018). Explanations for the relationship between elevation and species richness rely on
176 biological and ecological factors, and researchers have suggested comparing the richness
177 patterns between similar altitudinal ranges to obtain a more general pattern (Hodkinson, 2005;

178 Kaltsas et al., 2018).

179 For β diversity, understanding differences in species composition variations along elevational
180 gradients can help to determine what shapes community structure (Kraft et al., 2011) and
181 provides a reference for identifying conservation areas (Pereira Gomes et al., 2020). In the
182 context of montane ecosystems, changes in range size (Rodríguez and Arita 2004), habitat
183 filtering (Kaltsas et al., 2018) and species dispersal ability (Soininen et al., 2007) are thought
184 to be the main causes of turnover. For example, in the eastern Himalayas, the β diversity of
185 butterflies at adjacent sites peaked at mid-altitude, and the variations increased with distance
186 between sites, leading to the conclusion that the β diversity pattern was largely due to
187 environmental filtering (Dewan et al., 2022).

188 In addition to current patterns of species richness, the behavior of butterflies in response to
189 climate change has attracted widespread attention. As butterflies rely on the distributions of
190 host plants, changes in vegetation cannot be ignored. Severe destruction of low-elevation
191 habitats has been reported (Colwell et al., 2008). As temperatures become warmer, suitable
192 climatic conditions and vegetation will shift to higher elevations (Gottfried et al., 2012), and
193 butterflies respond to these environmental changes accordingly (Parmesan et al., 1999;
194 Macgregor et al., 2019; Rödder et al., 2021). In a study that analyzed 35 years of data from the
195 Sierra Nevada mountains, butterfly species richness decreased primarily at the lowest
196 elevations, with a significant upward shift in the range of butterfly species (Forister et al., 2010).

197 However, as recently reviewed, long-term monitoring of mountain butterflies is rare, especially
198 in the tropics (Halsch et al., 2021). Given that butterflies are adapted to particular habitats and
199 are sensitive to environmental changes, they have also been used to represent the rapid response

200 of animals to climate change over a relatively short period of time (Roth et al., 2014; Cerrato
201 et al., 2019). Monitoring butterflies has laid the foundation for evolutionary studies, especially
202 in the terms of migration, introgression, adaptation, and speciation.

203 In addition to surveys on species diversity, phylogenetic and population genetic studies have
204 also made remarkable progress in illustrating the genetic diversity, genetic structure, phylogeny,
205 and phylogeography of butterflies. For example, phylogeographic analyses have shown that
206 species diversity in *Melitaea* in the Middle Arctic originated in the Central Palaearctic during
207 the early Miocene, and a calibrated phylogeny based on sequences of *COI*, *EF-1 α* , and *Wingless*
208 has revealed the varying diversification rates of this genus, where the speciation rate became
209 faster with the ongoing orogenic process (Leneveu et al., 2009). This indicates that butterfly
210 biodiversity was shaped by paleoenvironmental changes. Coincidentally, *Erebia* is the most
211 diverse genus in the Palearctic, having diversified in response to paleoenvironmental changes
212 (Tennent, 2008). These butterflies depend on alpine habitats and thus show a fragmented
213 distribution across the Holarctic (Tennent, 2008). Further phylogenetic and biogeographic
214 analyses based on sequences of *COI*, *GAPDH*, *RpS5*, and *Wingless* reveal their origin in Asian
215 Russia, and the dispersal of the Western Europe lineage from Asia after the closure of the
216 epicontinental seaway was important for their radiation (Peña et al., 2015). Their speciation
217 process was probably driven by ecological specialization, such as flight time or the
218 differentiation of habitats (Kuras et al., 2000; Martin et al., 2002; Kleckova et al., 2014) and
219 allopatric speciation during Quaternary climatic oscillations (Vila et al., 2005; Albre et al.,
220 2008). Besides, genetic evidence based on sequences of *COI*, *ITS2*, and *RPS5* show that current
221 warming trend may have resulted in a decrease in the southern population of *E. orientalis*, while

222 a contrasting situation was observed in its young sister species *E. epiphron*, indicating their different
223 phylogeographical histories (Hinojosa et al., 2019). Such studies have contributed to a better
224 understanding of the formation and evolution of butterfly distributions, but rely on information
225 from a limited number of genetic loci. The contents and boundaries of these studies can be
226 expanded further as we gain more insight into butterfly genomic information.

227 **Box 1. Parameters of diversity**

228 Whittaker (1960) proposed a definition of species diversity that includes three levels of
229 measurement: α diversity, β diversity, and γ diversity, where α diversity and γ diversity represent
230 the number of species at a certain scale, with α diversity representing the number of species at
231 a local scale; thus, α diversity is also called within-habitat diversity. γ diversity reflects the
232 number of species at landscape scales and is mainly influenced by climate as well as by the
233 history of speciation and evolution. The Shannon–Wiener index and Simpson index are used to
234 measure diversity based on the number of species (Shannon, 1949; Simpson, 1949). The
235 definition of β diversity is more ambiguous, with the original definition by Whittaker being
236 "the extent of change in community composition, or degree of community differentiation, in
237 relation to a complex-gradient of environment, or a pattern of environments". Thus, β diversity
238 links local (α) diversity to greater (γ) diversity. Tuomisto (2010a, 2010b) thoroughly discussed
239 the interpretations of β diversity in different phenomena and explained what each of them
240 measures.

241

242 **Representative genetic and evolutionary models in butterfly research**

243 Studies of species richness and taxonomy provide a good basis for studying the evolutionary

244 scenarios and genetic mechanisms of biodiversity in mountain butterflies. Another important
245 basis for butterfly research may come from recent advances in genetics and genomics. In fact,
246 in addition to the vital role butterflies play in ecological networks, because of the simple
247 structure and complex functions of their wings that directly reflect selection factors, they have
248 been used as ideal study systems in evolution, genetics, development, physiology, and
249 behavioral biology (Beldade and Brakefield, 2002; Kronforst et al., 2015; Jiggins et al., 2017;
250 Reppert and de Roode, 2018). Next, we describe some relevant studies of butterfly genetics and
251 genomics, which have greatly broadened the field of butterfly research. The analytical tools
252 applied to omics data and experimental methods, not to mention the theoretical advances
253 obtained from these studies, will benefit research on the evolution and genetics of butterfly
254 diversity in the mountains.

255 Obtaining a reference genome is an important precondition to fuel genomic analysis. The first
256 butterfly genome was released in 2011 (Zhan et al., 2011). Some metrics are applied for
257 assessing the quality of genome assembly, such as N50 for continuity (the sequence length of
258 the shortest contig at half of the total assembly length) and BUSCO for completeness
259 (Benchmarking Universal Single-Copy Orthologs) (Waterhouse et al., 2018). High-quality
260 genomes offer opportunities for comparative genomics. For example, the synteny analysis
261 between the postman butterfly *Heliconius melpomene* and the silkworm *Bombyx mori* have
262 provided insights into the evolution of butterfly chromosomes (The *Heliconius* Genome
263 Consortium, 2012). The prediction of genes revealed expansion of chemosensory genes in
264 *Heliconius* (The *Heliconius* Genome Consortium, 2012), which may facilitate speciation (van
265 Schooten et al., 2020; Wu et al., 2022). Moreover, the genomes have laid the foundation for

266 functional genomics that is detailed in **Box 2** and illustrated in Fig. 2. To date, the number of
267 available butterfly genomes exceeds 800 (Ellis et al., 2021).

268 The advances in research methods also bring new life to classic topics. For example, eyespots,
269 a pattern on the wings of butterflies, are mostly found and intensively studied in the family
270 Nymphalidae and may have complex biological functions such as courtship recognition and
271 predator avoidance (Monteiro, 2014). In *Junonia coenia*, *Distal-less (Dll)*, a gene involved in
272 appendage development (Panganiban et al., 1994), was shown to be expressed in the eyespots
273 (Carroll et al., 1994). Similar results were later obtained in other butterfly species (Brakefield
274 et al., 1996; Monteiro et al., 2006). Recent work has shown that not only the *Distal-less* gene
275 but also the ancestral gene regulatory network, including *Dll*, *spalt (sal)*, and *Antennapedia*
276 (*Antp*), is co-opted in the formation of eyespots. Knocking out the cis-regulatory elements of
277 *Dll* and *sal* leads to the loss of eyespots, antennae, legs, and wings (Murugesan et al., 2022).
278 Studies on eyespots have greatly contributed to the understanding of the diverse phenotypes
279 that have evolved within a conserved developmental framework.

280 In addition to specific phenotypes, there are a number of butterfly taxa that are models for
281 evolutionary studies, which have captured a fair amount of interest, especially with the
282 development of analytical methods, such as the remarkable neotropical butterflies in the genus
283 *Heliconius* and swallowtail butterflies in the genus *Papilio*. By sequestering cyanogens from
284 host plants (Engler et al., 2000) or synthesizing aliphatic cyanogenic glycosides (Nahrstedt and
285 Davis, 1981, 1983), *Heliconius* butterflies become unpalatable to predators. Pollen feeding
286 (Gilbert, 1972), pupal mating behavior in some species and pronounced visual acuity (Zaccardi
287 et al., 2006) also make this genus unique. Moreover, the diversified Müllerian comimicry pairs

288 between distantly related species show deep convergence in wing patterns, which are important
289 clues for anti-predation (Merrill et al., 2012) and mating (Naisbit et al., 2001). Thus, the
290 identification of the genetic basis of mimicry has been the subject of considerable research. In
291 recent years, a few toolkit genes related to wing patterning have been characterized in
292 *Heliconius* butterflies (Reed et al., 2011; Martin et al., 2012; Nadeau et al., 2016; Van
293 Belleghem et al., 2017). These toolbox genes also play a role in wing patterning in other
294 butterflies, further showing reconciliation of developmental constraints and diversification. For
295 example, the *wntA* gene controls the Mendelian melanin switch in *Limenitis*, which mimics the
296 toxic species *Battus philenor* (Gallant et al., 2014), whereas the *optix* gene is involved in
297 pigmentary and structural coloration in *Agraulis vanilla*, *Vanessa cardui*, and *J. coenia* (Zhang
298 et al., 2017). Due to the ongoing process of speciation in *Heliconius*, much phenotypic diversity
299 can be recreated by interspecific hybridization (Gilbert, 2003). In addition, population genomic
300 analyses have helped clarify the phylogenetic relationship with genome-wide datasets (Van
301 Belleghem et al., 2017; Edelman, N.B. et al., 2021) and pointed to adaptive introgression in
302 *Heliconius*, which contributed to the understanding of wing pattern mimicry (The *Heliconius*
303 Genome Consortium, 2012; Pardo-Diaz et al., 2012; Zhang et al., 2016) and locomotion (Zhang
304 et al., 2021). Unlike studies on *Heliconius* butterflies, studies on *Papilio* butterflies have
305 focused on the Batesian mimicry of their wing patterns and have endeavored to reveal the
306 underlying genetic basis. These swallowtail butterflies show sexual dimorphism and limited
307 female mimicry polymorphism, meaning that females mimic a toxic model species, while males
308 display nonmimetic wing patterns (Joron and Mallet, 1998; Kunte, 2009a, 2009b). Crossover
309 experiments showed that the entire mimetic wing pattern is controlled by a Mendelian locus,

310 and then genomic analyses localized the control of this mimicry to the *doublesex* gene, which
311 is a key regulator of sex determination in insects (Kunte et al., 2014; Nishikawa et al., 2015).
312 Further analyses showed a common genetic basis for mimicry throughout *Papilio* butterflies
313 (Komata et al., 2016; Palmer and Kronforst, 2020), as well as a second loss of polymorphism
314 in the *Papilio polytes* species group (Zhang et al., 2017). Thus, butterflies have become a system
315 with well-developed genetic manipulation methods, as well as abundant multi-omic resources.
316 These studies have shown the value of butterfly studies and provided research schemes for
317 further investigations on mountain butterflies.

318 **Box 2. Methodological advances in the studies of butterfly traits**

319 The reference genome is the basis for conducting genomic analysis. As sequencing price
320 declines and methods of genome assembly develop, the assembled genomes of butterflies
321 become increasingly available
322 ([https://ncbi.nlm.nih.gov/genome/?term=txid37572\[Organism:exp\]](https://ncbi.nlm.nih.gov/genome/?term=txid37572[Organism:exp]),
323 <http://ensembl.lepbase.org/index.html>). The key to understanding the genetic basis of a
324 particular trait is to bridge the phenotypic variations to the sequence variations. The frequently
325 used methods for conducting genome-wide scans include but are not limited to genome-wide
326 association studies (GWAS) (Kunte et al., 2014; Wang et al., 2022), measuring divergence using
327 the fixation index (F_{ST}) (Van Belleghem et al., 2017), and scanning selective signals (Zhan et
328 al., 2014). For functional validation of candidate genes, multiple methods have been adopted
329 in butterfly studies. For example, *in situ* hybridization can be used to track gene expression by
330 combining dye molecules with targeting RNA in fresh tissues and is usually conducted in wing
331 discs of the late 5th instar stage (Martin et al., 2012; Martin and Reed, 2014; Nadeau et al.,

332 2016). RNA interference is another method acting at the transcription level. Double-stranded
333 RNAs can be introduced into an organism to dysfunctionalize the aligned RNAs. However,
334 many lepidopterans are strongly resistant to RNAi (Terenius et al., 2011; Kolliopoulou and
335 Swevers, 2014), which limits the application of this method. In recent years, with the
336 development of the CRISPR/Cas9 system, genome editing has become accessible in butterflies.
337 Using this method, it is possible to study the function of not only coding regions but also
338 regulatory regions, which reveals a deep convergence of employment of genes and their
339 regulatory elements among species (Mazo-Vargas et al., 2017, 2022; Zhang et al., 2017). In
340 addition to knockouts, knockins have also been achieved in butterflies (Zhang and Reed, 2017).

341

342 **Evolutionary studies of mountain biodiversity using butterfly genetics and genomics**

343 The above three sections described the unique biodiversity of mountainous regions and the use
344 of butterflies as evolutionary models. In recent years, studies have been conducted to integrate
345 the above research questions and research methods, such as advances in genomic analysis and
346 genome-editing methods, which have already benefitted studies on mountain butterflies. We
347 provide case studies from two perspectives. The first is provided to explore the mechanism of
348 unique biodiversity in mountains by integrating multiple dimensions. Multiple diversification
349 scenarios can be investigated based on genomic analyses at the level of species diversity,
350 whereas the functional basis underlying phenotypic variations can be characterized at the level
351 of genetic diversity. The second is about investigating the genetic mechanism of adaptation to
352 diverse habitats in mountainous areas, for example, the adaptation to high altitudinal
353 environments or the differentiation between mountain and lowland populations. Once the

354 candidate gene is characterized, genome-editing methods could come on the stage to carry out
355 functional validation. We will detail these two aspects with some examples in the following
356 paragraphs.

357 In *Kallima* butterflies, multiple species have been observed to co-occur in Medog, which
358 belongs to the eastern Himalayan region (Wang et al., 2022). Genome-wide demographic
359 analysis revealed that directional outward migration events have occurred in species from
360 Medog, and combined with ecological niche reconstruction results, the eastern Himalayas have
361 been proposed as the origin and diversification center of *Kallima* butterflies (Wang et al., 2022).

362 In this study, it was also found that *Kallima* butterflies maintain long-term trans-specific leaf
363 masquerade polymorphism, and the *cortex* gene was found to be involved in controlling this
364 phenotypic diversity based on genome-wide association studies (GWAS), which was further
365 validated by generating mosaic knock-out mutants using the CRISPR/Cas9 system (Wang et
366 al., 2022).

367 Studies on *Heliconius* butterflies are the pioneering research on adaptation to high mountains.
368 Butterflies in this genus are found from sea level to approximately 2,000 m, maintain a high
369 level of diversity in the Andes, with each species living within a characteristic altitudinal range
370 (Jiggins, 2018). *Heliconius* species adapted to higher altitudes are found to have rounder wings,
371 and in one clade of *Heliconius*, individuals possess larger wings than their lowland relatives
372 (Montejo-Kovacevich et al., 2019). By rearing progeny of *H. melpomene* and *Heliconius erato*
373 from across the cline in common garden conditions, the wing shape trait was found to be
374 inheritable. Further GWAS and selective signal scans helped to identify several genetic regions
375 that may shape wings, including several genes identified to influence wing morphogenesis in

376 *Drosophila* (Montejo-Kovacevich et al., 2021). Moreover, by sequencing populations from
377 transects across the mountain, repeated genetic differentiation was found within *H. melpomene*
378 or *H. erato* (Montejo-Kovacevich et al., 2022).

379 Some butterflies are distributed in both mountainous and lowland areas, but their populations
380 can have differentiations from each other in life history. Notably, the monarch butterfly *Danaus*
381 *plexippus* is famous for its long-distance migratory ability. Although most monarch butterflies
382 migrate annually from North America to central Mexico (east of the Rockies), populations
383 living in the west area of the Rockies overwinter along the California coast, and populations
384 dispersed elsewhere, such as in Hawaii, New Zealand and Ecuador, do not migrate (Dingle et
385 al., 2005; Lyons et al., 2012). By sequencing the genomes of 89 *Danaus* butterflies, researchers
386 identified northern migratory populations as the basal lineage, and further analysis of selective
387 signals uncovered that 5 Mb of the genome was associated with migration, and a 21-kb
388 fragment stood out as an outlier (Zhan et al., 2014). In this region, divergence of *collagen IV α -*
389 *I* was characterized as differentiating overwinter behavior, perhaps by reducing the flight
390 metabolic rate (Zhan et al., 2014).

391

392 **Conclusions and future perspectives**

393 Mountainous areas hold a unique combination of climates and geological conditions, which
394 provide diversified habitats for animals and plants, leading to high species richness. However,
395 with climate and land-use change, a loss of biodiversity has been observed across the globe
396 (Almond et al., 2022). Moreover, the drop in richness accelerates in biodiversity hotspots.
397 According to the 2022 Living Planet Report, which includes plant and animal species,

398 biodiversity in the American tropics has experienced the most significant decline (Almond et
399 al., 2022). This region is famous for its levels of species richness. At the same time, some
400 studies from China have also revealed a positive correlation between threatened species and
401 species richness (Lu et al., 2020). Therefore, more attention should be given to the dynamics of
402 mountain biodiversity. As an irreplaceable part of an ecosystem, insects are facing a rapid
403 decline, but the data available are inadequate (Troudet et al., 2017; Cardoso et al., 2020; Warren
404 et al., 2021). Butterflies, which are surveyed relatively often and are sensitive to environmental
405 changes, are good indicators and thus could be used better understanding insect declines.
406 Nevertheless, more comprehensive assessments of butterfly diversity patterns at a global scale
407 are still needed. In addition, comparisons between mountainous areas with similarities could be
408 conducted to obtain a general understanding of butterfly distribution patterns, if they exist.

409 Butterflies could not only be used for revealing current biodiversity distribution, but also help
410 us understand the origin of unique biodiversity in mountains. In-depth analysis of geological
411 and climatic events can help us better trace the evolutionary history of species and explain the
412 formation of rich diversity, and in some cases, studies of mountain biodiversity can help us
413 infer orogenic scenarios based on strong correlations between environmental and biological
414 changes. For example, researchers have found that the origin of herpetofauna in the Himalayas
415 dates back to the Paleocene but diversified rapidly during the Miocene. This conclusion
416 supports a stepwise geologic model of Himalayan uplift, which occurred during the Paleocene
417 with rapid uplift during the Miocene (Xu et al., 2021). Since most butterflies do not migrate
418 and are sensitive to environmental changes, butterfly demographics can also reflect geological
419 changes (Herrera-Alsina et al., 2021), especially in relatively young mountainous areas. The

420 fact that, compared with other insects, butterflies can be relatively easy to recognize and
421 identify in the field, as well as the fact that most butterflies possess medium-sized genomes
422 (Challis et al., 2016), makes them accessible for sampling and conducting molecular clock
423 analysis.

424 Furthermore, studies that documented patterns of species richness and phylogeny at local or
425 regional scales have laid essential foundations for further analysis of the evolution and genetics
426 of mountain butterflies. Advanced sequencing and analytical methods also provide additional
427 ways to understand the origin and evolution of mountainous species, and to reveal the
428 mechanisms of adaptation from a genetic perspective. For example, when climate changes,
429 shifts in mountain vegetation may isolate populations or create secondary contacts for poorly
430 differentiated populations, which provides an opportunity to study the speciation process.
431 Altitude variations along mountains or between mountains and lowlands, as well as niches
432 diversified vertically or between different sides of a mountain are good systems for studying
433 organismal adaptation and coevolution between hosts and insects. With the emergence and
434 development of butterfly genetics and genomics, we look forward to more research on mountain
435 butterflies to unravel the conundrum of unique mountain biodiversity, reveal the mechanisms
436 of endemic species emergence, and uncover the genetic basis of adaptation.

437

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444

445 **Conflict of interests**

446 The authors declare that they have no competing interests.

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447 **References**

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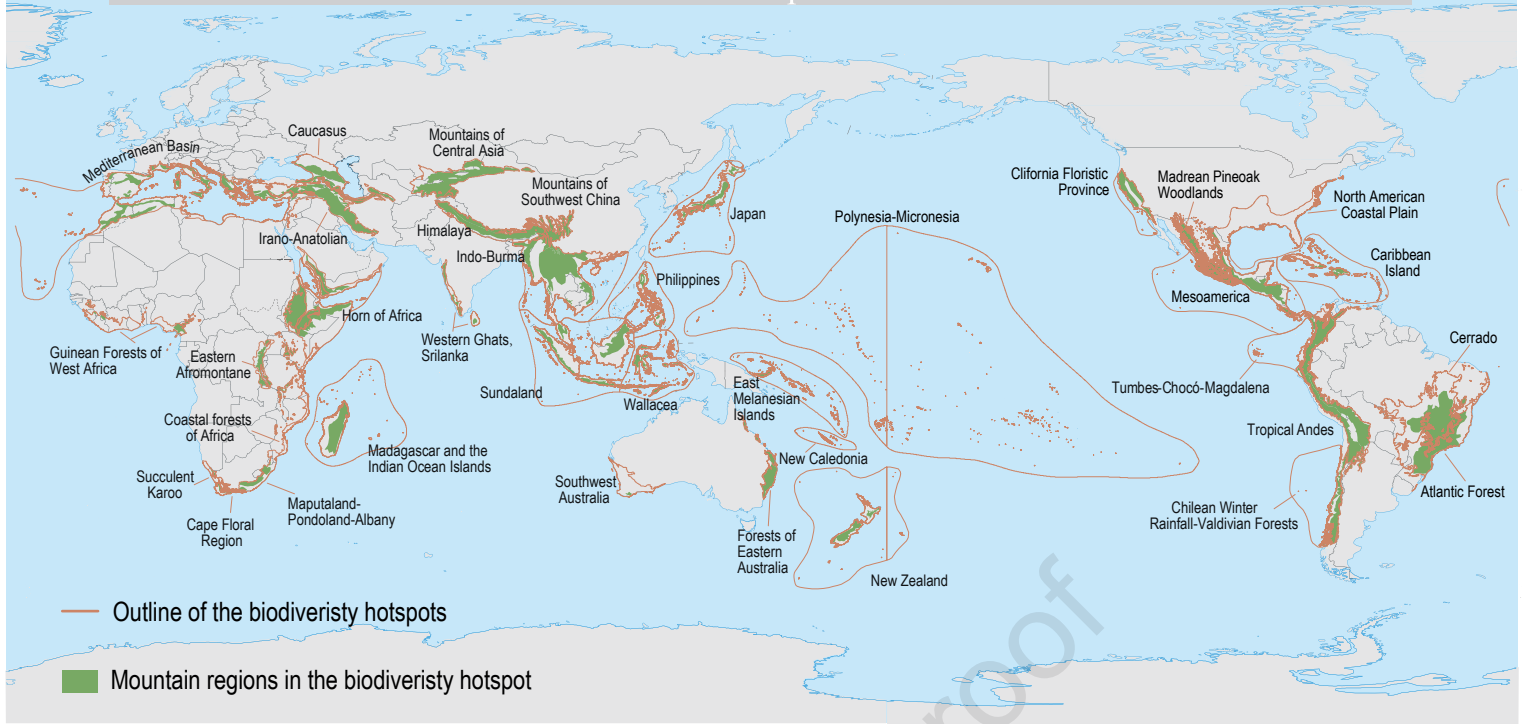
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840 **Figure legends**

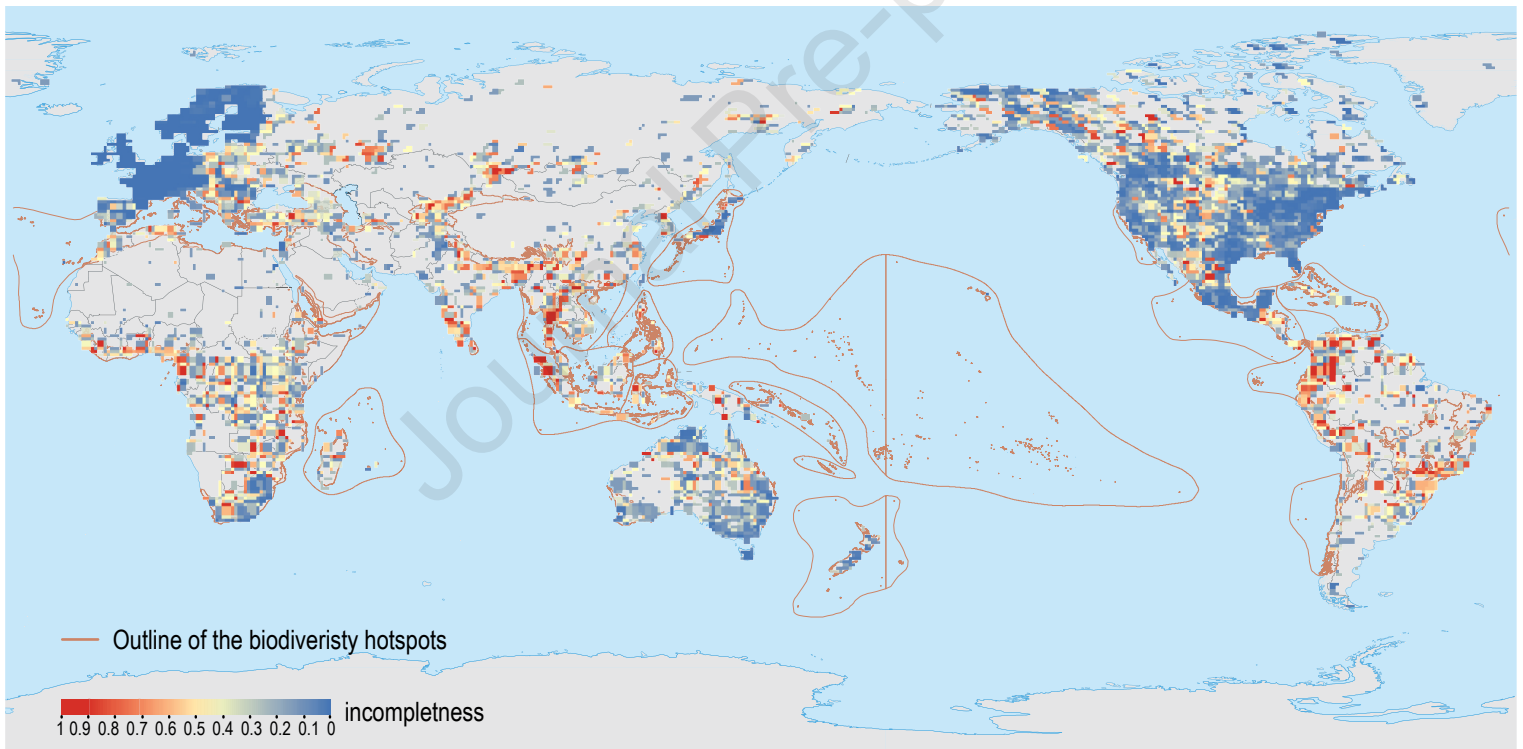
841 **Fig. 1.** Geographic distribution of mountainous biodiversity hotspots and the assessment of
842 global gaps in butterfly inventories. **A:** Mountainous regions in the biodiversity hotspots. The
843 biodiversity regions follow the definition by Myers et al. (2000), and the shapefile was
844 downloaded from Zenodo (Hoffman et al., 2016). Mountainous regions are as defined in
845 Rahbek et al. (2019c). **B:** Butterfly inventory incompleteness. Inventory completeness was
846 estimated using smoothed species accumulation curves (SACs) following Yang et al. (2013),
847 which tend to be straight in poorly sampled areas and highly curvilinear in better sampled areas.
848 The average slope of the last 10% of SACs indicates the degree of curvilinearity, which can
849 be used as a proxy for inventory completeness (Yang et al., 2013). Butterfly inventory
850 incompleteness was calculated as the slope of the last 10% of SACs for grid cells at a
851 resolution of 110 km. A value of 0 indicates complete inventory, while 1 indicates highly
852 incomplete inventory. The blank region means there is no available record from GBIF. The
853 base map was officially approved with number GS(2016)1665.

854 **Fig. 2.** Examples of dry lab and wet lab methods used in butterfly research. Several multi-
855 omics research techniques and experimental research tools have been applied to study
856 butterflies as a model, including but not limited to those illustrated in this figure, and these
857 methods have helped to reveal the evolutionary history of butterflies and the genetic
858 mechanisms of important traits.

A



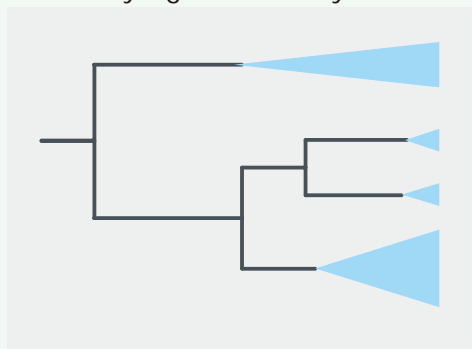
B



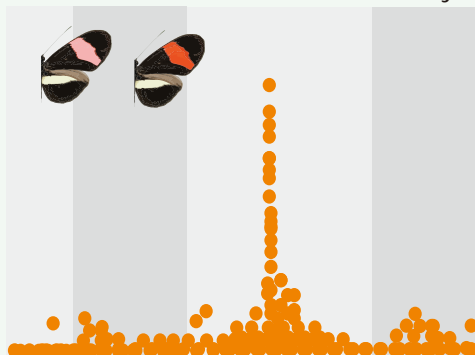
Dry lab



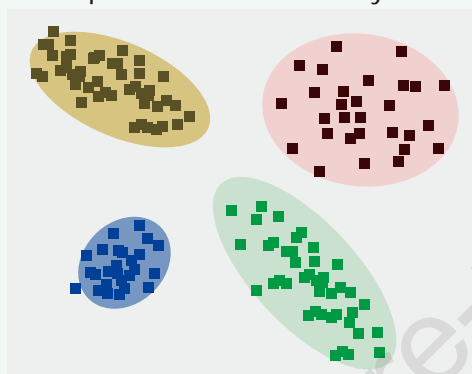
Phylogenetic analysis



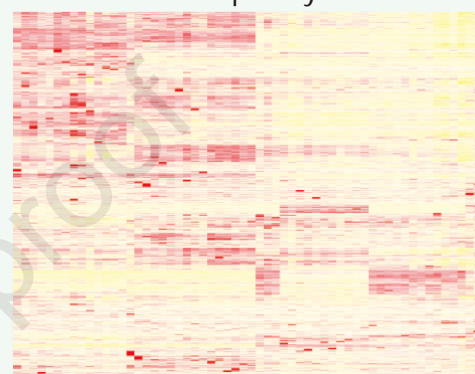
Genome-wide association study



Population structure analysis



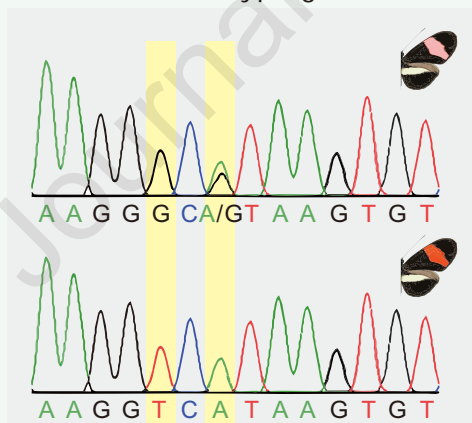
RNA-seq analysis



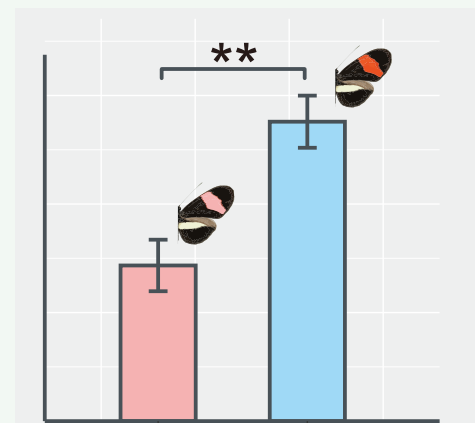
Wet lab



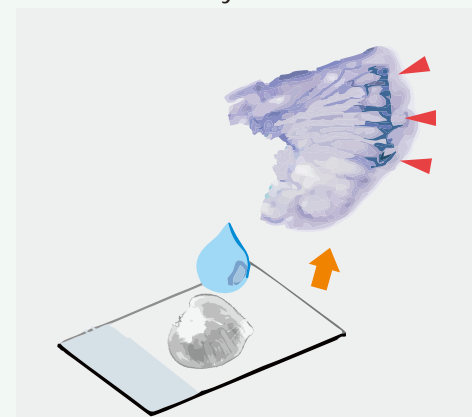
Genotyping



Quantitative PCR



In situ hybridization



CRISPR-Cas9 genome editing

